Nicotinamide Coenzyme Concentrations in Livers of Normal, Starved and Alloxan-Diabetic Rats

By D. S. KRONFELD and FIORA RAGGI
Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, Pa., U.S.A.

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Langdon (1957) demonstrated the role of reduced nicotinamide–adenine dinucleotide phosphate ($NADPH_2$) in fatty acid synthesis in rat liver, and suggested that the impairment of lipogenesis in diabetes may be attributable in part to a decreased availability of $NADPH_2$. This hypothesis won support at first (Siperstein & Fagan, 1958), and was extended by others to the lipogenic changes found in liver from rats deprived of food and then fed (Tepperman & Tepperman, 1958), and in mammary gland from spontaneously ketotic cows (Kronfeld & Kleiber, 1959). More recently it has been thought disproved with regard to diabetes (Matthes, Abraham & Chaikoff, 1960; Gordon, 1963).

The concentration of $NADPH_2$ estimated spectrophotometrically was found to be about the same as normal in diabetic-rat liver (Glock & McLean, 1955*b*). Using a fluorimetric method, we found a much lower than normal concentration of $NADPH_2$, and of the other nicotinamide dinucleotides, in mammary biopsy samples from spontaneously ketotic cows (Kronfeld & Raggi, 1964). The fluorimetric assay has now been applied to livers of starved and diabetic rats.

METHODS

Rats. Males of the Wistar strain, body weight 200–225 g., were given Purina Laboratory Animal Chow at 7 a.m. daily. Some feed remained available up till the time of killing (11 a.m. till noon) for rats in the normal group. The starved rats were deprived of food for 48 hr. Diabetes was induced with alloxan; the rats were starved for 24 hr., then injected intraperitoneally with freshly prepared alloxan solution at a dose of 180 mg./kg. Polyuria, glycosuria and ketonuria developed in 2–7 days in 13 out of 17 rats so treated. Glycosuria was detected by Uristix (Ames Co., Elkhart, Ind., U.S.A.), which is a paper strip impregnated with glucose oxidase, peroxidase and *o*-tolidine, which turns dark purple in a glucose solution of more than 0·5 mg./100 ml. Ketonuria was detected by a nitroprusside test (Dumm & Shipley, 1946).

The rats were decapitated with large shears. Mixed blood from the severed head vessels was collected for the estimation of glucose concentration (Campbell & Kronfeld, 1961). At the same time, the abdomen was slit open, five pieces of liver (about 400 mg. each) were removed and placed on a Petri dish frozen on ice. Four of the samples were weighed on a Mettler H5 balance, placed in acid or alkaline extraction solutions (Bassham, Birt, Hems & Loening, 1959) at 0°, and the test tubes put into the water baths to begin the assays. This procedure took 2 min. from the time of decapitation. The fifth liver sample was assayed for protein by a biuret method (Gornall, Bardawill & David, 1949).

Coenzyme assays. We followed the method of Bassham *et al.* (1959). It depends on the selective destruction of oxidized forms by alkali and of reduced forms by acid, and on the specificity of alcohol dehydrogenase (EC 1.1.1.1) for NAD and of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) for NADP. The modifications needed for mammary gland (Kronfeld & Raggi, 1964) were not needed for liver. The only changes from the original method of Bassham *et al.* (1959) were the incubation of the alkaline (reduced) fractions at 60° for 10 min., and the use of a Turner fluorimeter model 111, primary filter 7–60 and secondary 75 (G. K. Turner Associates, Palo Alto, Calif., U.S.A.).

Preparations of NAD, $NADH_2$, NADP and $NADPH_2$ were purchased (Sigma Chemical Co., St Louis, Mo., U.S.A.) and standardized as before (Kronfeld & Raggi, 1964). Known amounts were added to ice-cold extraction solutions before the addition of weighed samples from adjacent parts of the liver. Recoveries were: NAD, 96%, $NADH_2$, 100%, NADP, 101%, and $NADPH_2$, 89%. The precision of our assays was calculated by analysis of variance of 25 duplicate assays (Table 1). According to the *F* test (Table 1), the precision was adequate for discriminating differences between rats for NAD, $NADH_2$ and $NADPH_2$, but not for NADP. This was not due to a lower precision in the assay of NADP (Table 1), but rather to a lower variance in the NADP concentration between rats. Also, according to the recent report of Burch, Lowry & VonDippe (1963), there may have been loss of NADP in our procedures, as well as those of previous investigators [listed by Burch *et al.* (1963), by Caiger, Morton, Filsell & Jarrett (1962) and by Lindall (1962)].

RESULTS

The ranges of blood glucose concentration were 85–118 mg./100 ml. in eight normal rats, 50–105 mg./100 ml. in eight starved rats, and 200–370 mg./100 ml. in nine diabetic rats, with 1235 mg./100 ml. in the tenth diabetic rat. The protein concentrations in the liver were about the same in all three groups: means (±s.e., mg. of protein/g. wet tissue) were 236 ± 11 (normal), 229 ± 13 (starved) and 238 ± 6 (diabetic).

The means and S.E. of the concentrations of NAD, $NADH_2$, NADP and $NADPH_2$ and of the ratios NAD/$NADH_2$ and NADP/$NADPH_2$ are set out in Table 2. The normal values are similar to those found by several other investigators (see Table 1 of Caiger *et al.* 1962), except that our values for NADP are higher than those of most other workers and lower than those of Lowry's group (Caiger *et al.* 1962; Burch *et al.* 1963).

Compared with normal values, NAD was significantly decreased and $NADH_2$ increased, so that the ratio NAD/$NADH_2$ was decreased during food deprivation. Also, the increase in $NADPH_2$ concentration was not sufficient to change the ratio NADP/$NADPH_2$ significantly. During diabetes, the only change in concentration was a decrease in $NADPH_2$, and this raised the ratio NADP/$NADPH_2$ significantly (Table 2).

These results have been compared with previous assays of these coenzymes in livers of starving and diabetic rats (Table 3). Similar data from a number of other reports (reviewed by Lindall, 1962) have not been included in this comparison because some conditions were different and/or insufficient numbers of animals were studied to allow statistical analysis. Despite differences in detail, it appears in Table 3 that the significant changes from normal concentrations are: a decrease

Table 1. *Precision of coenzyme assays*

Standard deviation of duplicate assays on 25 livers was calculated by analysis of variance. Adequacy of precision was evaluated by F, the ratio of the estimated variance between assays to the variance within assays.

Coenzyme	S.D. within assays (μm-moles/g. of fresh tissue)	Percentage of mean	F	P
NAD	46	13·8	9·53	<0·001
$NADH_2$	38	15·6	5·85	<0·001
NADP	22	27·8	1·60	<0·2
$NADPH_2$	28	11·2	15·64	<0·001

Table 2. *Nicotinamide coenzymes in rat liver*

Fluorimetric assays (modified from Bassham *et al.* 1959) were done on two liver samples from each rat. Significance of differences of starving and diabetic means from the normal means are assessed by the t test: *, $P < 0.05$; **, P 0·01; ***, P 0·005; ****, P 0·001.

	Normal	Starving (48 hr.)	Diabetic
No. of rats	8	8	10
Concentrations (μm-moles/g. wet wt. mean ± S.E.M.)			
NAD	407 ± 25	223 ± 19****	363 ± 22
$NADH_2$	219 ± 16	296 ± 17**	222 ± 24
NADP	79 ± 6	77 ± 9	79 ± 7
$NADPH_2$	271 ± 17	321 ± 13*	176 ± 18***
All	977 ± 42	917 ± 41	840 ± 54
Ratios (arithmetic mean ± S.E.M.)			
NAD/$NADH_2$	1·90 ± 0·46	0·76 ± 0·16****	1·79 ± 0·66
NADP/$NADPH_2$	0·30 ± 0·07	0·24 ± 0·07	0·47 ± 0·13***

Table 3. *Deviations from normal in the concentrations and ratios of nicotinamide coenzymes in diabetic- and starving-rat liver*

Significant changes in the means ($P < 0.05$ or better) are expressed as percentages of the corresponding normal mean, with decreases indicated by minus signs. —, No significant change.

	NAD	$NADH_2$	NADP	$NADPH_2$	NAD/$NADH_2$	NADP/$NADPH_2$
Diabetic-rat liver						
This paper	—	—	—	−35	—	+57
Glock & McLean (1955*b*)	−20	—	—	—	−43	—
Lindall (1962)	—	+48	+94	−23	−42	+150
Starving-rat liver (48 hr.)						
This paper	−45	+35	—	+18	−60	—
Glock & McLean (1955*b*)	−48	—	—	+108	−36	—
Lindall (1962)	—	+46	—	—	−52	—

in NAD and increases in $NADH_2$ and $NADPH_2$ in starved-rat liver, but only a decrease in $NADPH_2$ in diabetic-rat liver.

DISCUSSION

The different changes in concentrations of the nicotinamide dinucleotides found in livers from diabetic and starving rats suggest that the lipogenic defects in these two conditions are different from one another, at least with respect to the roles played by these coenzymes. If this is the case, the diabetic defect, but not the starving defect, might in part be due to a low concentration of $NADPH_2$. Alternatively, if the causes of the two lipogenic defects are identical, then they would be independent of these coenzyme concentrations. Consistent with the assumption of identical causes of the two defects are similar losses of liver enzyme activities [acetyl-CoA carboxylase (EC 6.4.1.2) (Numa, Matsuhashi & Lynen, 1961; Wieland & Eger-Neufeldt, 1963) and the long-chain acyl-CoA synthetase system (Gibson & Hubbard, 1960)] in both starvation and diabetes. But Korchak & Masoro (1962) have shown that it is likely that loss of these enzyme activities during food deprivation is a secondary effect rather than the initiating cause of the lipogenic defect, and Wieland & Eger-Neufeldt (1963) have differentiated a second stronger defect (additional to the decrease in acetyl-CoA carboxylase activity) in acute diabetes with severe ketosis. Thus the decrease in acetyl-CoA-carboxylase activity found in food deprivation and in diabetes may be only a part of the total lipogenic defect in each case. It is possible that the initial cause of the lipogenic defects may be the same in each case, but that divergence occurs with time as the lesion becomes more complex. For the remainder of this discussion we will assume the causes of the lipogenic defects operating at the time of the coenzyme assays were not entirely the same in the starving as in the diabetic rats.

In diabetic-rat liver, the finding of a significantly lower than normal concentration of $NADPH_2$ is consistent with the original hypothesis of Langdon (1957), which attributed this lipogenic defect in part to decreased availability of the coenzyme. Only in part, however, for a diminution of about 30 % in the coenzyme concentration seems hardly likely to account for all of the diminution in lipogenic activity, which is often over 70 % (Abraham, Migliorini, Bortz & Chaikoff, 1962; Gordon, 1963). Clearly, the rate of lipogenesis is influenced by additional factors, e.g. activation by substrate, citrate (Brady & Gurin, 1952; Spencer & Lowenstein, 1962; Vagelos, Alberts & Martin, 1963), and inhibition by product, palmitoyl-CoA (Porter & Long, 1958; Tubbs, 1963; Bortz & Lynen, 1963*a*).

The rate of generation of $NADPH_2$ may come closer to the meaning of 'availability' than does concentration. The activities of two enzymes which generate $NADPH_2$, glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and phosphogluconate dehydrogenase (EC 1.1.1.43), expressed per gram of liver, were depressed by about 60 % in alloxan-diabetic rats (Glock & McLean, 1955*a*). In contrast, these hepatic enzyme activities were about the same in depancreatized rats and in controls when both groups were given 60 % glucose, and in both were nearly four times those of normal rats on a stock diet (Abraham *et al.* 1962). Siperstein & Fagan (1958) found that adding $NADPH_2$-generating systems (NADP plus glucose 6-phosphate or plus citrate) to diabetic-rat liver homogenates partially restored their lipogenic activity, and concluded that a deficiency of $NADPH_2$ was the specific cause of the lipogenic defect. Since the restoration was only partial, their experiments suggest that a $NADPH_2$ deficiency might be one but not the only causative factor. $NADPH_2$ generation estimated from the $^{14}CO_2$ production after adding specifically labelled glucose 6-phosphate and citrate to normal and diabetic-rat-liver homogenate fractions was about the same in both preparations (Matthes *et al.* 1960; Abraham *et al.* 1962). They concluded that $NADPH_2$ generation from these substrates by the diabetic-rat-liver preparations under conditions optimum for fatty acid synthesis was not impaired. The optimum conditions of their reconstituted and fortified preparations might not, however, be present in the intact diabetic-rat-liver cell. Another method of estimating $NADPH_2$ generation has been presented by Gordon (1963). Labelled nicotinamide was injected intraperitoneally into rats and the specific activities of hepatic nicotinamide dinucleotides were determined. 'Turnover times' calculated for control and diabetic rats were 8·1 and 3·5 min. respectively. Gordon concluded that the rate of $NADPH_2$ generation was greater than normal during diabetes. The amount or pool size of the $NADPH_2$ was not reported (Gordon, 1963) and, if it is not known, the shorter turnover time could just as readily be interpreted as a sign of decreased pool size as of increased generation rate. It seems to us that the present information on the availability of $NADPH_2$ to the lipogenic system(s) of diabetic-rat liver is equivocal.

In starving-rat liver, the finding of a significantly higher than normal concentration of $NADPH_2$ would not be consistent with a deficiency of this coenzyme contributing to this lipogenic defect. Nor would the evidence on the rate of $NADPH_2$ generation: the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, again expressed per gram of liver, were

scarcely affected by restricted food intake (Glock & McLean, 1955*a*), and the ratio of the specific activities of NADP and $NADPH_2$ after labelled nicotinamide was given to unfed rats was indistinguishable from controls (Gordon, 1963).

The higher than normal concentration of $NADPH_2$ in starving-rat liver suggests its accumulation, which would be consistent with a lipogenic defect due to some other cause. Korchak & Masoro (1962), upon finding that declines in the activities of acetyl-CoA carboxylase and the palmitate-synthetase system were less severe and slower to develop than the decline in lipogenesis when food was withheld, suggested that the failure of lipogenesis is the cause of loss of these enzymes rather than an effect. Masoro, Korchak & Porter (1962) described cytoplasmic particles from liver of starved rats which inhibited fatty acid synthesis by the supernatant system. Bortz & Lynen (1963*a*) have shown that long-chain acyl-CoA derivatives inhibit acetyl-CoA carboxylase, and that they accumulate in the liver of starved rats (Bortz & Lynen, 1963*b*). Such a lipogenic defect due initially to the appearance of inhibitors and then to a loss of the fatty acid-synthesizing enzymes would be expected to lead to the accumulation of $NADPH_2$.

SUMMARY

1. The concentrations and ratios of nicotinamide coenzymes has been estimated by a fluorimetric method in liver samples from normal, starved and alloxan-diabetic rats. The assay had adequate precision to discriminate differences between animals in NAD, $NADH_2$ and $NADPH_2$, but not NADP.

2. Compared with normal, NAD concentration was significantly lower and $NADH_2$ higher during starvation, but not during diabetes. $NADPH_2$ was lower during diabetes but higher during starvation. The ratio $NAD/NADH_2$ was decreased during starvation but not diabetes, and $NADP/NADPH_2$ was increased during diabetes but not starvation.

3. The results suggest that the lipogenic defects during food deprivation and in alloxan-diabetes are different, at least with respect to the roles played by the nicotinamide dinucleotide coenzymes. If this is the case, the low $NADPH_2$ concentrations found in diabetic-rat liver would be consistent with this lipogenic defect being partly due to a diminished availability of this coenzyme. The high $NADPH_2$ concentration found in starved-rat liver is consistent with its accumulation due to loss of enzymes which utilize it.

4. Alternatively, if all the causes of the total lipogenic defect which develops during food deprivation and in diabetes are identical, they would not include changes in the concentrations of the nicotinamide adenine dinucleotides.

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